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We have focussed our efforts on investigating the mechanisms by which antisense to type I insulin-like growth factor receptor (IGF-IR) inhibits cell growth and reduces tumorigenicity in human metastatic breast cancer cells. MDA-MB-435s breast cancer cells carrying antisense IGF-IR displayed decreased expression of endogenous IGF-IR, reduced cell proliferation, loss of clonogenicity and enhanced apoptosis *in vitro*. There was a dramatic suppression in tumorigenesis and an absence of lung metastases in both nude and scid beige mice injected with MDA-MB-435s cells carrying antisense IGF-IR. All animals injected with the control cells carrying the construct minus the antisense IGF-IR insert developed large tumors. The scid beige mice consistently exhibited pulmonary metastases whereas there were no apparent metastatic sites in the lungs of nude mice. We provide data that shows that the length of survival in scid beige mice injected with MDA-MB-435s cells carrying the antisense IGF-IR was dramatically increased compared to animals injected with cells carrying the construct minus the antisense IGF-IR insert. These results demonstrate that the IGF-IR plays a critical role in the progression of metastatic estrogen receptor negative human breast cancer. Therapies directed toward inhibiting the expression of this receptor may provide a new treatment strategy for patients with progressive metastatic breast cancer.

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INTRODUCTION:

Breast cancer kills around 50,000 women each year in North America with world figures about 10-fold higher (Lopez-Otin and Diamandis, 1998). The incidence of breast cancer has been steadily increasing while the mortality rates for breast cancer have not changed significantly for the last 50 years (Hortobaggi and Buzdar, 1995). A major characteristic of tumor malignancy and the primary cause of mortality for patients with breast cancer is the ability of the tumor cells to metastasize to distant sites. Breast cancer mortality is related to the tumor cell's capacity to invade, metastasize and proliferate (Leis, 1991). Later stages of breast cancer involve metastasis to bone, brain and other organs. Indeed, ninety percent of patients dying with breast cancer have osteolytic bone metastases (Yoneda et al., 1994). Advanced breast cancer often no longer responds to conventional therapies, therefore it is urgent to find treatments that will result in an enhanced survival and ultimately a cure. We have applied our experience in treating and preventing rat glioblastoma (Trojan et al., 1993), murine teratocarcinoma (Trojan et al., 1994) and rat prostate carcinoma (Burfeind et al., 1996) to develop a model for human metastatic breast cancer.

To assign a role of a gene product, one relies on naturally occurring mutants that fail to express the gene in normal fashion. Generation of cellular mutants has limited applicability due to the diploid nature of most genes and the lack of adequate mutant selection. Our experimental approach for breast cancer centers around the use of antisense RNA expression to produce phenocopies of a null mutation of the type I insulin-like growth factor receptor. The antisense strategy bypasses inherent limitation of functional studies dependent upon natural mutant cells or artificially mutagenized cells. Inhibition of IGF receptors by antisense provides a direct approach for assessing their contribution to the tumorigenic and metastatic phenotype of cancer cells both in culture and *in vivo*. The antisense RNA will either hybridize to the endogenous mRNA or disrupt it transcription or processing, thereby preventing the synthesis of protein product (Izant and Weintraub, 1985). We (Johnson et al., 1991) and others (Keiss et al., 1989) have reported that rat glioma C6 cells express high levels of IGF-I which is enhanced when the cells are grown in serum-free medium. We have observed that rats injected with transfected glioma cells that express antisense IGF-I RNA and which lack IGF-I protein continue to remain tumor free for more than two years. In contrast, rats injected with parental (nontransfected) glioma cells consistently develop large tumors within a few weeks post injection. These data demonstrate a transformation role for IGF-I, highlighting the fact that transformed phenotypes may arise through unexpected molecular mechanisms. It represents a clear example of a dominant and essential role for a growth factor in malignant transformation.

Antisense strategy has been applied successfully to a growing set of genes in both cultured cells and transgenic animals (Katsuri et al., 1988; Munir et al., 1990). However, the antisense approach has frequently been complicated by incomplete inhibition of gene expression (Munir et al., 1990; Khokha et al., 1989). We (Johnson et al., 1991; Trojan et al., 1992) have demonstrated effective antisense inhibition of endogenous IGF-I transcripts in C6 glioblastoma cells by Northern analysis and inhibition of protein by immunocytochemistry. Antisense transcripts were prominently localized to the nuclei by *in situ* hybridization supporting an intranuclear mechanism for antisense RNA mediated inhibition, in agreement with observations

1988). A finding in our study was accumulation of mononuclear infiltrates, with a predominance of lymphoid cells at sites of injection of antisense IGF-I transfected glioma cells before lesions disappeared (Trojan et al., 1992). The majority of cells were CD8 positive, suggesting antisense IGF-I inhibition rendered glioma cells highly immunogenic, and hence, loss of tumorigenicity had an immune basis. This is further supported by a study that shows that glioblastoma cells transfected with antisense to IGF-I demonstrate a significant increase in major histocompatibility complex-I (MHC-I) when compared to untransfected cells (Trojan et al., 1996; Shevelev et al., 1997). We have shown that prior injection of the antisense IGF-I transfected glioma cells can prevent development of glioblastoma upon subsequent challenge with parental glioma cells. Injection of the genetically engineered glioma cells into rats with established glioblastomas cures the rats. The findings are consistent wit a role for the host immune response in the anti-tumor therapeutic effects (Trojan et al., 1993; Johnson et al., 1993).

Prior research has focused on the potential role of the IGFs as growth factors driving the proliferation of tumor cells. This has led to attempts to inhibit growth of tumor cells with anti-IGF-I or anti-IGF-II antibodies. While some growth inhibition has been claimed using these antibodies *in vitro* (Minuto et al., 1987; Huff et al., 1986; Blatt et al., 1984) and *in vivo* (Gansler et al., 1989), tumor development could not be completely blocked. Moreover, since intracrine mechanisms (Heldin and Westermark, 1989) may well be involved here, there is no certainty that the effects of anti-IGF-I antibodies will parallel those of antisense IGF-I RNA in alter tumor immunogenicity in an immunologically intact animal.

Tumors that arise *de novo* are poorly immunogenic, thereby escaping host anti-tumor responses (Hewitt et al., 1979). Our studies provide a potential therapeutic approach toward enchancing tumor immunogenicity based upon antisense gene transfer. Alternative approaches based upon sense gene transfer have been reported for enhancing tumor cell immunogenicity (Fearon et al., 1988). However, loss of tumorigenicity of transfected tumor cells and tumor prevention using these cells were incomplete. The second approach involves enhancement of tumor immunogenicity by transferring into cells, genes expressing soluble cytokines, such as interleukin-2 (Fearon and Vogelstein, 199) and interleukin-4 (Tepper et al., 1989; Golumbek et al., 1991). Results with this approach have been more promising than those with foreign antigen transfer. Indeed, it has been demonstrated that human breast cancer cells MDA-MB-435 transduced with human IL-2 did not form tumors when injected into the mammary fat pad of nude mice (Su et al., 1994). Furthermore, it was shown that transfecting cultured murine melanoma cells with the co-stimulator B7 evoked an effective immune response that results in regression of the existing tumor in syngeneic animals (Townsend and Allison, 1993; Chen et al., 1992).

Many primary tumors and cell lines from tumors produce large amounts of IGFs and IGF receptors (Antoinades et al., 1992; Roholl et al., 1990; Williams et al., 1989; Gansler et al., 19880 Culouscou et al., 1987; Macaulay et al., 1990; Jing et al., 1991). Most important for this proposal are breast carcinomas that have been shown to express IGF receptors (Yee et al., 1988; Foekins et al., 1989). Cullen et al. (1991) examined breast cancer cell lines and tumor samples for mRNA expression of the insulin receptor as well as the type I and type II IGF receptors. All cell lines examined by this group expressed mRNA for these receptors. In addition, 6 of 7 breast tumor biopsy specimens expressed type I IGF receptor mRNA. The monoclonal antibody

(αIR3) which blocks binding to the type I IGF receptor also blocked the mitogenic effects of both IGF-I and IGF-II, but not insulin. The αIR3 antibody was also able to block greater than 80% of radiolabeled IGF-II binding. Furthermore, αIR3 administered at the time of tumor cell inoculation could inhibit MDA-231 tumor formation in athymic mice which suggests that blockade of the IGF-IR can suppress the growth of some breast cancer cells *in vivo* (Arteaga et al., 1989; Arteaga, 1992). This indicates that although both type I and type II IGF receptors are expressed in breast cancer cells, the mitogenic response to both IGF-I and IGF-II is mediated by the type I receptor (Cullen et al., 1990). Most breast tumors express IGF receptors, therefore it is important to ascertain whether the inhibition of IGF-IR gene expression by an antisense approach can alter the tumorigenic and metastatic potential of breast cancer cells. For this reason, we have carried out studies to determine the effect of blocking the expression of IGF-IR using antisense strategy in the highly metastatic human breast cancer cell line, MDA-MB-435s. We focussed our efforts on blocking the expression of the IGF-IR based on a report from our laboratory (Rininsland et al., 1997). This study showed that IGF-I was suppressed in C6 rat glioblastoma cells transfected with either antisense to IGF-IR or by an IGF-IR purine triplex expression vector. These data suggested that one common mechanism could account for decreased tumorigenicity of the C6 cells regardless of whether IGF-I or IGF-IR expression is inhibited. The inhibition of IGF-I by suppression of IGF-IR by two independent approaches indicates that by targeting IGF-IR it is possible to inhibit the expression of the ligand as well as the receptor. Therefore, it is possible that targeting the IGF-IR in breast cancer cells with antisense to the receptor could also inhibit the expression of endogenous ligands such as IGF-I and IGF-II.

The type I insulin-like growth factor receptor (IGF-I) has been shown to play a central role in the mechanism of malignant transformation (Baserga, 1995). Reports have demonstrated that the IGF-IR is present on cells from several human breast cancer cell lines (Furlanetto and DiCarlo, 1984; Cullen et al., 1990). The important role of IGF-IR in beast cancer is supported by data from several laboratories. Previous studies have reported that IGF-IR levels are significantly higher in tissue from breast cancer than in normal breast tissue or benign tumors (Lee et al. 1995; Peyrat and Bonneterre, 1992; Jammes et al., 1992). Patients with tumors containing a high IGF-IR gene copy number tend to have a shorter median overall survival than patients with tumors having a low amplified IGF-IR gene copy number (Berns et al., 1992). Indeed, a recent study has shown that IGF-IR expression was 14 fold higher and that IGF-IR autophosphorylation and kinase activity were 2-4 fold higher in malignant breast tissue than in normal breast tissue (Resnik et al., 1998). This amounts to a 40-fold elevation in IGF-IR tyrosine kinase activity in malignant breast tissue. Moreover, breast cancer cells appear to possess certain intrinsic properties that facilitate the development of bone metastases. Almost all patients dying of breast cancer or with advanced breast cancer have bone metastases (Yoneda et al., !994). Breast cancer cells may prefer bone to other organs because of the growth factors in the microenvironment. Bone has been shown to be a major source of IGFs, with humans having the highest concentration of total skeletal somatomedins of all species studies (Bautista et al., 1990). Indeed, IGF-II is the most abundant growth factor stored in bone matrix.

A study has demonstrated that hepatic tumor cells expressing IGF-IR antisense RNA lost their ability to metastasize spontaneously to the liver or lung from primary subcutaneous tumors and could not colonize these organs, even when inoculated directly into their

microvasculature (Long et al., 1995). These results implicate IGF-IR in the control of tumor growth and show that IGF-IR can play a crucial role in the regulation of tumor cell potential to disseminate and form metastases in secondary organs. Therefore, targeting breast tumor cells with an antisense IGF-IR strategy could provide an effective antimetastatic therapy for this disease. A recent report demonstrated that elevated circulating concentrations of the ligand for IGF-IR, insulin-like growth factor I show a strong association with the relative risk of breast cancer in premenopausal women (Hankinson et al., 1998). The overexpression of IGF-IR seen in some breast cancer cells coupled to elevated concentrations of circulating IGF-I may contribute to the progression of the cells to a malignant phenotype. Therefore, it is urgent to develop treatments that will alter the cascade that leads to metastases by targeting growth factors and their receptors.

BODY:

In the fourth and final year of this project, we have continued to make progress in several of the proposed goals as well as in the areas that we presented in our previous progress reports. We have focussed our efforts on investigating the effectiveness of antisense IGF-IR in reducing tumorigenicity and metastasis of the human breast cancer cells, MDA-MB-435s. Moreover, we have completed an extensive series of experiments on this highly metastatic human breast cancer cell line.

The aim of the experiments on the MDA-MB-435s estrogen receptor negative human breast cancer cells was to assess the effect of treatment by an antisense IGF-IR gene targeting approach. The objectives were to evaluate the role of IGF-IR in cell growth, apoptosis, tumorigenesis and metastases.

We established by Northern blot analysis that the MDA-MB-435s cells avidly express the type I insulin-like growth factor receptor. We successfully transfected the cells with our antisense IGF-IR construct and identified several cell clones that displayed high expression of antisense IGF-IR for further analysis. The two clones, C8 and C9 with the greatest level of antisense IGF-IR expression were used to further assess the role of IGF-IR in cell growth, apoptosis, tumor formation and metastases.

The two MDA-MB-435s human breast cancer cell clones showed strong expression of antisense IGF-IR by Northern analysis of poly A^+ RNA (Figure 2 A, Chernicky et al., Appendix I). The expression of endogenous IGF-IR by the MDA-MB-435s cells carrying antisense IGF-IR was reduced by 43% in the cells from the C8 clone and by 56% in cells from the C9 clone (Figure 2C, Chernicky et al., Appendix I).

The proliferation rates of the cells carrying antisense IGF-IR were significantly inhibited on days 3 and 5 when compared to the proliferation rate of control cells carrying the construct minus the antisense IGF-IR insert (Figure 3A, Chernicky et al., Appendix I).

Anchorage-independent growth in the semisolid medium of soft agar is a strong indicator of the transformed phenotype and a more stringent test of mitogenic capacity. Colony formation is soft agar was completely abrogated in cells from the clones carrying the antisense IGF-IR construct (Figures 4A and 4B, Chernicky et al., Appendix I). In contrast, cells carrying the sense IGF-IR (Figure 4C, Chernicky et al., Appendix I) or the construct minus the inserts (Figure 4D, Chernicky et al., Appendix I) exhibited avid clonogenic growth.

A decrease in the number of IGF-IRs by treatment with an antisense approach has been shown to trigger massive apoptosis (programmed cell death) in several types of cancer cells. We found that cells carrying antisense IGF-IR from the C8 and C9 cell clones were apoptotic using two different methods, DNA ladder analysis and flow cytometry. Cells from both clones exhibited DNA laddering on days 3 and 5 of serum withdrawal (Figure 5, Chernicky et al.,

Appendix I). Flow cytometric analysis carried out on cells from the C9 clone indicated that approximately 59% of the cells were apoptotic (Figure 6, Chernicky et al., Appendix I).

To assess whether treatment of the MDA-MB-435s human breast cancer cells with antisense IGF-IR affects the tumorigenic potential of the cell *in vivo*, assays were carried out in female athymic mice. A dramatic inhibition in tumorigenesis was observed in the mice injected with cells from either the C8 or C9 clone (Figure 7, Chernicky et al., Appendix I). In addition the animals injected with cells from the C8 or C9 clone displayed a significant delay in onset of tumor formation and inhibition in tumor size compared to tumor from mice injected with cells carrying the construct minus the IGF-IR inserts (Figure 8, Chernicky et al., Appendix I).

To establish whether the rudimentary immune system that exists in nude mice (deficient in T cells) plays a role in the develop of tumors a bioassay using nude and scid beige mice (deficient in T, B and NK cells) was carried out. All of the animals that were injected with MDA-MB-435s cells carrying the construct minus the IGF-IR inserts developed large tumors. In contrast, tumor size was dramatically reduced in all animals injected with cells from the C8 or C9 clones when compared to mice injected with MDA-MB-435s cells transfected with the control vector (Figure 9, Chernicky et al., Appendix I). There were no apparent metastatic sites in lungs from mice injected with cells from the C8 or C9 antisense IGF-IR expressing clones. On the other hand, in animals injected with the cells carrying the construct minus the antisense IGF-IR insert all of the scid beige mice had metastases to the lungs at the time of sacrifice (Table I, Chernicky et al., Appendix I). This data suggests that the rudimentary immune system plays a role in suppression metastases in nude mice and indicates that the scid beige mouse model may be the most suitable for studying the metastatic potential of these human breast cancer cells.

An additional bioassay using scid beige mice was performed to investigate the long term effects on tumorigenesis following treatment with an antisense IGF-IR approach. The survival curve (Figure 10, Chernicky et al., Appendix I) shows that animals injected with cells from the C8 or C9 clone had a significantly enhanced survival. In contrast, all of the animals injected with cells carrying the construct minus the IGF-IR inserts die or need to be sacrificed within 3 months of receiving the cells. Overall the animals injected with cells from the C9 clone showed the greatest increase in survival. The increased survival appears to correlate with the level of anitsense expression and the degree of suppression of endogenous IGF-IR as shown in Figure 2 (Chernicky et al., Appendix I).

CONCLUSIONS:

The long term goal of this study was to develop a gene therapy treatment to cure human breast cancer. Our ultimate aim was to develop a database supporting the hypothesis that certain growth factors and their receptors are involved in the tumorigenicity of some breast cancers. The objectives of our studies have been to develop model systems in which to analyze the effect of blocking the expression of these growth factors and their receptors using antisense expression plasmids that we prepared.

We focussed our studies in this fourth and final year on the role of the type I insulin-like growth factor receptor in tumorigenesis and metastases. We have presented data that clearly shows that the IGF-IR plays a critical role in cell growth, tumorigenesis and metastases in a highly metastatic human breast cancer cell line, MDA-MB-435s. Breast cancers that become hormone independent (loss of estrogen receptors) are more likely to metastasize and indicate a poorer prognosis for the patient. Therefore the results of our experiments show that the development of therapies that target the IGF-IR could provide a new treatment approach for certain types of aggressive metastatic breast cancer.

We have also demonstrated that it is extremely important to establish the optimal animal model to study metastases. Our results comparing nude mice to scid beige mice indicate that the rudimentary immune system present in nude mice has an inhibitory effect on the development of pulmonary metatases from tumors caused by injection of MDA-MB-435s cells.

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Appendix I

TREATMENT OF HUMAN BREAST CANCER CELLS WITH ANTISENSE RNA TO THE TYPE I INSULIN-LIKE GROWTH FACTOR RECEPTOR INHIBITS CELL GROWTH, SUPPRESSES TUMORIGENESIS, ALTERS THE METASTATIC POTENTIAL AND PROLONGS SURVIVAL *IN VIVO*

Running title: Antisense IGF-IR inhibition of breast tumor growth. Cheryl L. Chernicky M.T.[*], Lijuan Yi B.S.[*], Huiqing Tan M.S.[*], Shu Uin Gan Ph.D.[*1] and Judith Ilan Ph.D.[*]

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Key Terms: cell transfection, MDA-MB-435s cells, animal models, metastases

ABSTRACT

The type I insulin-like growth factor receptor (IGF-IR) plays an important role in the growth and transformation of breast cancer cells. In this study we investigated the effects of treatment with an antisense IGF-IR construct on cells from the highly metastatic estrogen receptor negative human breast cancer cell line MDA-MB-435s. The cells carrying the antisense IGF-IR had a markedly reduced expression of IGF-IR, a significant decrease in cell proliferation and lost the ability to form colonies in soft agar. There was a delay in tumor formation and a dramatic reduction in tumor size when cells carrying the antisense IGF-IR were injected into either nude or scid beige mice. We have also provided data that showed that the scid beige mouse is a more suitable model for studying metastasis of the MDA-MB-435s cells. All of the scid beige mice injected with cells carrying the control construct had metastasis to the lungs while lungs from the nude mice had no apparent metastatic sites after 11 weeks. When cells carrying antisense IGF-IR were injected s.c. in scid beige mice the animals had a significant increase in survival compared to mice injected with cells carrying the control construct. These results taken together show that IGF-IR can play a critical role in the progression of estrogen receptor negative breast cancer cells. Our studies provide a basis for development of future treatment strategies targeting the IGF-IR in estrogen receptor negative metastatic breast cancer.

INTRODUCTION

Breast cancer kills around 50,000 women each year in North America with world figures about 10-fold higher.[1] The incidence of breast cancer has been steadily increasing while the mortality rates for breast cancer have not changed significantly for the last 50 years.[2] A major characteristic of tumor malignancy and the primary cause of mortality in patients with breast cancer is the ability of the tumor cells to metastasize to distant sites. Breast cancer mortality is related to the tumor cell's capacity to invade, metastasize and proliferate.[3] Later stages of breast cancer involve metastasis to bone, brain and other organs where conventional treatments are often no longer effective. Therefore it is important to define the role of the genes that are responsible for the progression of breast cancer cells to the metastatic phenotype and develop treatment strategies that target these genes.

The type I insulin-like growth factor receptor has been shown to play a central role in the mechanism of malignant transformation.[4] The presence of the IGF-IR on cells from several breast cancer cell lines has been demonstrated by competitive-binding studies and affinity cross-linking as well as by RNase protection assays.[5,6] Several studies have reported that IGF-IR levels are significantly higher in tissue from breast cancer than in normal breast tissue or benign tumors.[7,8,9] Patients with tumors containing a high IGF-IR gene copy number tend to have a shorter median overall survival than patients with tumors having a low amplified IGF-IR gene copy number.[10] Indeed, a recent study has shown that IGF-IR expression was 14 fold higher and that IGF-IR autophosphorylation and kinase activity were 2-4 fold higher in malignant breast tissue than in normal breast tissue.[11] This amounts to a 40-fold elevation in IGF-IR tyrosine

kinase activity in malignant breast tissue. It has also been previously demonstrated that the use of αIR3, an antibody specific for IGF-IR inhibited cell proliferation and suppressed tumor growth of MDA-MB-231 human breast cancer cells.[12]

Studies from several laboratories including our own have used antisense strategies that target IGF-IR to show inhibition of tumor growth for melanoma[13], glioblastoma[14], human lung cancer cells[15], rhabdomyosarcoma[16], osteosarcoma[17], mesothelioma[18] and prostate cancer[19,20]. In addition a recent study from our group has shown that inhibition of IGF-IR expression in rat glioblastoma cells can be brought about by using a triple helix strategy.[21] It has also been reported that MCF-7 human breast cancer cells transfected with a construct carrying antisense for IGF-IR RNA displayed a reduced growth rate in culture conditions.[22] On the other hand a recent study has shown that over expression of the IGF-IR promotes aggregation, growth and cell survival in MCF-7 human breast cancer cells.[23] Therefore our hypothesis was that IGF-IR played a role in human metastatic breast cancer and that cancer cells treated with a stable antisense IGF-IR expressing construct have a decreased probability of forming metastases.

In this study, we have applied an antisense strategy targeting the type I insulin-like growth factor receptor to a metastatic estrogen receptor negative human breast cancer cell line, MDA-MB-435s. We provide data that demonstrates the efficacy of this approach for inhibiting growth of these highly metastatic human breast cancer cells *in vitro* as well as *in vivo*. We have also shown the importance of establishing and using the optimal animal model for assessing tumor growth and the metastatic potential of cells in vivo. Moreover, we have observed that transfection of MDA-MB-435s human breast cancer cells with an antisense IGF-IR construct can significantly inhibit the incidence of

lung metastases and prolong survival when these cells are injected into animals.

MATERIALS AND METHODS

Cell Line and Cell Culture. Human breast cancer cells, MDA-MB-435s were purchased from American Type Culture Collection (Rockville, MD). These highly metastatic human breast cancer cells were derived from the parent MDA-MB-435 cell line.[24] This cell line originated from a pleural effusion in a 31-year-old female with metastatic, ductal adenocarcinoma of the breast. No systemic therapy was given to the patient prior to establishing the cell line. The cells were maintained in Dulbecco's modified Eagle medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, UT). Culture medium for transfected MDA-MB-435s cells was supplemented with geneticin at 500 μg/ml (Life Technologies Inc., Gaithersburg, MD) to maintain selection pressure. Before injection into 4-6 week old female athymic nude or scid beige mice the cells (2×10^6) were washed once in PBS, treated with Versene 1:5000 (Life Technologies Inc., Gaithersburg, MD) for 3-5 min. and resuspended in PBS.

Construction of Vectors. To assemble the pRcII/CMV vector used in these experiments a pRc/CMV vector (Invitrogen, Carlsbad CA) was modified by removing DNA sequences from nucleotide position 995 (*Apa*I site in the multiple cloning site) to nucleotide position 2100 (*Sma*I site upstream of the neomycin resistance gene). Resulting DNA fragments were separated by agarose gel electrophoresis. A 4.3 kb vector fragment was isolated with a US Bioclean kit (United States Biochemical Corp., Cleveland, OH) and religated with T4 DNA ligase (Boehringer Mannheim Corp.,

Indianapolis, IN). Following transformation the modified vector pRcII/CMV was grown in *E. coli* and then purified by using a plasmid isolation kit (Qiagen Inc., Chatsworth, CA). The human IGF-IR cDNA 697 bp fragment (nucleotide position 42 in exon 1 to nucleotide position 738 in exon 3, Genbank accession no. M24599) that was obtained from total RNA isolated from T47D human breast cancer cells by RT-PCR (described in Burfeind et al., 1996) was cloned into the *Hin*dIII/*Eco*RI sites of the pRcII/CMV vector in the antisense orientation (Figure 1A). To assemble the sense vector the 697 bp IGF-IR cDNA fragment was cloned into the EcoRI/*Pst*I sites of the plasmid pBluescript II SK (Stratagene, La Jolla, CA) which was then cloned into the *Hin*dIII/*Not*I sites of the pRcII/CMV vector (Figure 1B). The directional cloning of the sense and antisense IGF-IR cDNA inserts was confirmed by restriction mapping.

Transfection. MDA-MB-435s human breast cancer cells were transfected with the antisense IGF-IR vector, the sense IGF-IR vector or with the pRcII/CMV control vector using Lipofectin (Life Technologies Inc., Gaithersburg , MD) according to the supplier's instructions. Geneticin (G-418 Sulfate)(Life Technologies Inc., Gaithersburg, MD) at a concentration of 1 mg/ml was used to select for cells that were neomycin resistant indicating that the vector was present in the cells. Several (n=11) individual cell clones were isolated from the population of cells carrying the antisense IGF-IR vector. All of the transfected cells and cell clones were maintained in DMEM with 10%FBS and G418 (0.5mg/ml).

Northern Blot Analysis. Total RNA was isolated from cells with Trizol reagent (Life Technologies Inc., Gaithersburg MD). Poly A^+ RNA was then selected using the Messagemaker reagent assembly (Life Technologies Inc., Gaithersburg MD) according to

the manufacturer's instructions. Samples (5μg Poly A^+ RNA or 20 μg of total RNA/sample) were electrophoresed on a 1% denaturing agarose gel followed by transfer to a Hybond-N nylon membrane (Amersham Lifesciences Inc., Arlington Hts, IL). The cDNA probes, a 0.7 kilobase fragment from the human IGF-IR sequence and a 2.2 kb fragment of chicken β-actin were labeled with α-[^{32}P]dCTP (DuPont NEN Research Products, Boston MA) using the random hexanucleotide primer method.[25] Northern blot hybridization was carried out in 5 X SSC, 5 X Denhardt's solution, 10% (wt/vol) dextran sulfate, 0.1% (wt/vol) SDS and 100 μg/ml denatured salmon sperm DNA at 65°C for 18 h. After hybridization the membrane was washed for 15 minutes at room temperature with 2 X SSC followed by a final 15 minute wash in a solution consisting of 0.5 X SSC and 0.5% SDS (wt/vol) at 65°C. The membranes were exposed to x-ray film of 6-12 h. X-ray films were analyzed with a SciScan 5000 laser densitometer (United States Biochemical Corp., Cleveland OH) and normalized relative to β-actin mRNA.

Proliferation Assays. The tetrazolium salt (MTT) method involving conversion of MTT to colored formazan by cells serves an as indirect measurement of cell growth and is an accurate reproducible means of measuring cell viability. Therefore a modified MTT assay was used to determine cell proliferation rates in MDA-MB-435s human breast cancer cells from clones C8 and C9 carrying antisense IGF-IR, cells transfected with sense IGF-IR or cells transfected with the control vector.[26] Cells were seeded into each well (2.5 X 10^3 cells/well) of 96 well plates. MTT (20μl, 5mg/ml in PBS) (Sigma Chemical Co., St. Louis, MO) was added on day 0, day 1, day 3 or day 5 of culture. The reaction was stopped after 1 hour of incubation at 37°C by addition of 100 μl of solubilization buffer (20%SDS (w/v) in 50% dimethylformamide; pH 5). Plates were then incubated at 37°C

for 4 to 16 hours. An enzyme-linked immunosorbent assay reader (Dynatech MR 5000; Dynatech Laboratories, Inc., Chantilly, VA) was used to measure absorbance at a wavelength of 570 nm and a reference wavelength of 690 nm. The reported cell numbers were extrapolated from a standard curve generated for each cell line and experiment relating absorbance to a known cell number. Data points are represented as the mean ±SE of six wells. All assays were repeated several times.

Soft Agar Colony Forming Assay. For soft agar assays, a bottom layer of 1 ml of the corresponding culture media containing 0.6% agar (Difco Laboratories, Detroit, MI) and 10% FBS was prepared, placed in 35 mm culture dishes (Corning Glass Works, Corning, NY) and allowed to solidify. Cells (2.5×10^3) from the C8 and C9 clones carrying antisense IGF-IR, cells carrying sense IGF-IR or cells transfected with the vector minus the inserts were suspended in 50 µl of DMEM containing 10% FBS and 0.5 mg/ml G418. Culture media (1 ml) containing 0.3% agarose (Boehringer Mannheim Corp., Indiananpolis, IN). was added to the cell suspension prior to seeding on the dishes. Triplicates were performed for each type of cell. Cells were incubated at 37°C in 5% CO_2 atmosphere. Dishes were examined twice a week and colonies were counted manually after 4 weeks.

DNA Fragmentation Assay for Apoptosis. Cells (1×10^6) from the MDA-MB-435s clones C8 and C9 as well as cells that were transfected with the control vector were seeded in 15ml of DMEM containing10% FBS and 0.5 mg/ml G418. On the third day of culture the medium was replaced with serum free DMEM. The cells were then maintained in serum free media for 3 or 5 days. Non-adherent cells and adherent cells treated with trypsin were combined. Genomic DNA was isolated from the combined

cells using a Promega Genomic DNA isolation kit (Promega, Madison, WI) according to the manufacturer's protocol. Genomic DNA (4 μg) and the molecular weight marker λDNA/*Eco*RI + *Hin*dIII (Boehringer Mannheim Corp., Indianapolis IN)(0.25 μg) were radiolabeled by *Taq* polymerase.[27] To label with *Taq* polymerase, the DNA was added to 20 μl of labeling mixture with a final concentration of 10 mM Tris-HCl pH 9.0, 50 mM KCl, 0.1% Triton X-100, 10 mM $MgCl_2$, 2μCi α^{32}P-dATP (3000Ci/mmol), and 1 U *Taq* polymerase. Reactions were incubated at 72°C for 20 min and terminated by the addition of gel loading buffer. The labeled DNA was electrophoresed on a 1.5% agarose gel. The gel was then exposed to x-ray film for 5 min at room temperature to visualize the bands.

TUNEL Analysis. Cells (1 X 10^6) from the MDA-MB-435s C9 clone and cells transfected with the control vector were seeded in 15 ml of DMEM containing 10% FBS and 0.5 mg/ml G418 in T-75 flasks (Falcon). On day 3 of culture the medium was replaced with serum free DMEM. Cells were harvested on the fifth day of serum withdrawal. Adherent cells were trypsinized and combined with non-adherent cells. The cells were then washed and adjusted to 1-2 X 10^7 cells/ml with PBS. A volume of 100μl of the cell suspension was taken from each sample and 100μl of freshly prepared paraformaldehyde solution (4% in PBS, pH 7.4) was added to the cell suspension. Samples were incubated for 30 min at room temperature on a shaker followed by centrifugation at 300 x g for 10 min. The fixative was removed and the cells were washed with 200 μl of PBS and centrifuged at 300 x g for 10 min. Cells were then resuspended in 100 μl of permeabilisation solution (0.1% Triton X-100 in 0.1% sodium citrate) for 4 min at room temperature. After permeabilisation the cells were washed 2x in PBS (200 μl) and further processed using an In Situ Cell Death Detection Kit, Fluorescein

(Boehringer Mannheim, Indianapolis, IN). Briefly, the cells were resuspended in 50 µl of TUNEL reaction mixture and incubated for 60 min at 37°C in the dark. The samples were washed 2x in PBS (200 µl/wash) and the cells were transferred to a tube (Falcon 2063) to a final volume of 250 µl in PBS. The negative control was carried out in a similar manner with the label solution (without terminal transferase) instead of TUNEL reaction mixture. For the positive control the cells were treated with DNase I (10 U/ml, 10 min at 37°C) to induce DNA strand breaks before labeling with TUNEL solution. The samples were analyzed by flow cytometry using an Elite ESP (Coulter Corp., Miami FL). Excitation was at 488 nm with emission read in the BP 525/25. Data were acquired using logarithmic amplification of the FITC signal. Data were analyzed using Elite Acquistion software.

Assay of Tumor Growth and Metastases in Animal Models. Female balb/c nude mice, 4-6 weeks of age and scid beige mice, 4-6 weeks of age were used. Nude mice were bred and maintained in the athymic animal facility at Case Western Reserve University School of Medicine, Cleveland OH. Scid beige mice were purchased from Charles River Laboratories (Wilmington, MA) and maintained in the athymic animal facility at Case Western Reserve University. The MDA-MB-435s human breast cancer cells from clones C8 and C9 carrying the antisense IGF-IR construct or the control cells carrying the construct minus the antisense IGF-IR insert were injected through a 22 gauge needle over the right scapula of the mice. All cells were injected in a volume of 100 µl at a concentration of 2×10^6 viable cells. The tumor take rate when the control cells were injected in the scapular region was 100%. Tumors were measured every week with vernier calipers. Tumor volume was calculated by the formula: volume = $(\text{width})^2 \times$

length/2. Animals were sacrificed after 11 weeks and the tumors excised and weighed. The lungs were removed, rinsed in PBS and placed in Bouin's solution for 24 hours prior to counting the number of metastatic sites. For the survival curve animals were sacrificed when the tumor size was greater than 10% of the animal's weight or when the tumor ulcerated.

RESULTS

The aim of the series of experiments carried out in this study was to assess the effect of treatment by an antisense IGF-IR gene targeting approach on cells from the estrogen receptor negative human breast cancer cell line, MDA-MB-435s. The objectives were to evaluate the role of IGF-IR in cell growth, apoptosis, tumorigenesis and metastases in this highly metastatic human breast cancer cell line.

After transfection of the MDA-MB-435s human breast cancer cells with a vector containing the IGF-IR cDNA in an antisense direction, several clones (n=11) carrying the antisense IGF-IR construct were identified and maintained in culture. In addition MDA-MB-435s cell populations carrying the sense construct or the control vector (construct minus the IGF-IR inserts) were identified. Northern blot analysis was carried out on all of the clones to select the cell populations that displayed the highest level of expression of antisense IGF-IR (Data not shown). Two clones, C8 and C9, with the greatest level of antisense IGF-IR expression were used to further assess the role of IGF-IR in cell growth, apoptosis, tumor formation and metastases.

The two MDA-MB-435s human breast cancer cell clones, C8 and C9 showed strong expression of antisense IGF-IR by Northern analysis of poly A^+ RNA (Figure 2A,

Lanes 2 and 3). The level of expression for the 11 kb principal transcript for IGF-IR was significantly decreased in cells from the C8 and C9 clones (Figure 2A, Lanes 2 and 3) compared to the level of IGF-IR expression by the control transfected cells (Figure 2A, Lane 1). Densitometric evaluation of the Northern blots indicated that expression of endogenous IGF-IR was reduced by 43% in cells from the C8 clone (Figure 2C, *stippled bar*) and by 56% in cells from the C9 clone (Figure 2C, *white bar*) relative to the level of expression of IGF-IR in cells transfected with the control vector (Figure 2C, *black bar*). The filter was rehybridized with a cDNA probe for chicken β-actin to verify the integrity and amount of poly $(A)^+$ RNA in the samples (Figure 2B).

A series of assays were performed to characterize the antiproliferative effects following treatment of MDA-MB-435s cells with the antisense IGF-IR construct. The proliferation rate of the cells from the C8 (Figure 3A, *triangles*) and C9 (Figure 3A, *circles*) clones carrying the antisense IGF-IR construct was significantly inhibited on days 3 and 5 when compared to the proliferation rate of control cells carrying the construct minus the IGF-IR inserts (Figure 3A, *circles*). To determine whether the inhibition was specific for the MDA-MB-435s cells transfected with the antisense IGF-IR construct an additional assay was carried out with MDA-MB-435s cells transfected with the sense IGF-IR construct. Cells carrying sense IGF-IR (Figure 3B, *squares*) showed a proliferation rate similar to that of cells transfected with the control vector (Figure 3B, *diamonds*) whereas cells from the C9 clone (Figure 3B, *circles*) carrying the antisense IGF-IR construct had a markedly decreased cell proliferation rate on days 3 and 5. In addition to slower growth rates, significant numbers of non-adherent cells were present in cultures of the C8 and C9 cell clones carrying the IGF-IR antisense construct compared

to the control transfected cells or cells carrying the sense IGF-IR construct (data not shown).

Anchorage-independent growth in the semisolid medium of soft agar is a strong indicator of the transformed phenotype and a more stringent test of mitogenic capacity because several cycles of cell division are required to form detectable colonies. Therefore, a series of soft agar assays was performed to assess the ability of human breast cancer cells treated with antisense IGF-IR to form colonies in soft agar. Colony formation in soft agar was completely abrogated in cells from the C8 and C9 clones carrying the antisense IGF-IR construct (Figure 4A and 4B, respectively). However, cells carrying the sense IGF-IR (Figure 4C) or the construct minus the inserts (Figure 4D) exhibited avid clonogenic growth under identical culture conditions.

A decrease in the number of IGF-IRs by treatment with an antisense approach has been shown to trigger massive apoptosis (programmed cell death) in several types of cancer cells.[28] Therefore, to determine whether MDA-MB-435s human breast cancer cells carrying antisense IGF-IR were apoptotic two independent methods were used, a DNA fragmentation laddering assay and FACS analysis. In the first set of experiments a DNA fragmentation assay was performed using cells from the C8 and C9 clones carrying antisense IGF-IR as well as cells transfected with the control vector. The characteristic ladder that forms when genomic DNA is electrophoresed on standard agarose gels shows that cells are undergoing apoptosis. Figure 4 shows a DNA ladder analysis carried out on the C8 and C9 clones carrying antisense IGF-IR. Cells from the C8 and C9 clones exhibited DNA laddering on day 3 of serum withdrawal (Figure 5, Lanes 3 and 5, respectively) with more prominent laddering occurring on day 5 of serum withdrawal

(Figure 5, Lanes 4 and 6, respectively). No appreciable evidence of apoptosis could be detected on either day 3 or day 5 of serum withdrawal in cells carrying the vector minus the IGF-IR inserts (Figure 5, Lanes 1 and 2 respectively).

The induction of DNA fragmentation was also confirmed by using a TUNEL assay. TUNEL is a sensitive and quantitative method for the detection of 3'-OH ends generated by endonuclease-mediated DNA strand breaks.[29] Figure 6 shows a flow cytometric analysis of cells from the C9 clone carrying antisense IGF-IR. There is a peak on the histogram (Figure 6A, *right panel*) that indicates that approximately 59% of the cells from the C9 clone carrying antisense IGF-IR are apoptotic. On the other hand the MDA-MB-435s cells carrying the construct minus the antisense IGF-IR insert (Figure 6B, *right panel*) display a peak similar to the negative control (Figure 6B, *left panel*) indicating that there is no evidence of apoptosis in these cells.

To assess whether treatment of the MDA-MB-435S human breast cancer cells with antisense IGF-IR affects the tumorigenic potential of the cells *in vivo,* assays were carried out in female athymic mice. The mice were injected with cells from the antisense IGF-IR C8 or C9 clones or control cells carrying the construct minus the IGF-IR inserts. A dramatic inhibition in tumorigenesis was observed in the mice injected with cells from either the C8 or C9 clone (Figure 7A and 7B, respectively). On the other hand the animals injected with cells carrying the construct minus the antisense IGF-IR insert developed large tumors (Figure 7C). Figure 8 shows that the nude mice injected with cells from the C8 or C9 clone displayed a significant delay in onset of tumor formation and a dramatic inhibition in tumor size compared to tumors from mice injected with cells carrying the construct minus the IGF-IR inserts. Three out of four mice injected with

cells from the C8 clone remained tumor free at the end of 11 weeks. All mice injected with cells from the C9 clone developed tumors, however the tumors were significantly smaller than tumors formed following injection of control cells.

To establish whether the rudimentary immune system that exists in nude mice (deficient in T cells) plays a role in the develop of tumors a bioassay using nude and scid beige mice (deficient in T, B and NK cells) was carried out. All of the mice that were injected with the MDA-MB-435s cells carrying the construct minus the IGF-IR inserts developed tumors. This is most likely due to the fact that the scapular region where the cells were injected houses the first and second thoracic subcutaneous mammary fat pads in the mouse and therefore provides an orthotopic site for growth of the breast cancer cells.[30] The tumor size was dramatically reduced in all animals injected with cells from the C8 (Figure 9, gray bars) or C9 (Figure 9, white bars) clones when compared to mice injected with MDA-MB-435S cells transfected with the control vector (Figure 9, black bars). Animals injected with cells from the C8 or C9 antisense IGF-IR expressing clones showed significantly reduced tumorigenicity with no apparent metastases to the lungs (Table 1). However, in animals injected with the cell carrying the construct minus the antisense IGF-IR insert only the scid beige mice had metastases to the lungs at the time of sacrifice. This suggests that the rudimentary immune system plays a role in suppressing metastases in nude mice and indicates that the scid beige mouse model may be the most suitable for studying the metastatic potential of these human breast cancer cells.

An additional bioassay using scid beige mice was performed to investigate the long term effects on tumorigenesis following treatment with an antisense IGF-IR approach. The survival curve shows that cells from both the C8 (Figure 10, *long dashes*)

and C9 (Figure 10, *short dashes*) clones carrying antisense IGF-IR resulted in a significantly prolonged survival for the animal. On the other hand all of the animals injected with cells carrying the construct minus the antisense IGF-IR insert (Figure 10, *solid line*) die or need to be sacrificed within 3 months of receiving the cells. Overall the animals injected with cells from the C9 clone showed the greatest increase in survival. The animals injected with cells from the C8 clone also had a significant increase in survival but not as dramatic as the animals injected with cells from the C9 clone. The increased survival appears to correlate with the level of antisense expression and the degree of suppression of endogenous IGF-IR as shown in Figure 2.

DISCUSSION

Our data provide support for the functional role of the type I insulin-like growth factor receptor in tumorigenesis and metastasis of the human breast cancer cells, MDA-MB-435s. We used an antisense gene therapy strategy that targets the IGF-IR to define mechanisms that are involved in the growth and progression of this metastatic estrogen receptor negative human breast cancer cell line. Studies have reported that there is a highly significant correlation between a high IGF-IR expression and better prognosis.[31,32] On the other hand several small series studies showed an association with a shorter disease free survival when the tumors were estrogen receptor negative and IGF-IR positive and that high IGF-IR levels correlated with resistance to radiotherapy.[33,34] The MDA-MB-435s human breast cancer cell line was used in these studies since loss of the estrogen receptor often indicates a poorer prognosis for the patient.[35] We carried out a series of experiments to determine whether down-regulation of IGF-IR by an antisense

approach could change the phenotype of these metastatic estrogen receptor negative human breast cancer cells.

We extended the findings of others that showed that targeting the IGF-IR by both monoclonal antibodies directed against IGF-IR and antisense strategies inhibit breast cancer cell growth *in vitro*. The monoclonal antibody α IR3 has been used to block the type I insulin-like growth factor receptor and successfully inhibit the growth of the estrogen receptor negative human breast cancer cells, MDA-MB-231.[36] An antisense strategy directed against IGF-IR has been used to suppress IGF-IR receptor expression in MCF-7 human breast cancer cells.[22]

The current data show that the IGF-IR is a requirement for growth of the estrogen receptor negative MDA-MB-435s human breast cancer cells. In this study we reported that MDA-MB-435s human breast cancer cells carrying an antisense IGF-IR construct exhibited a decrease in expression of IGF-IR at the mRNA level. The reduction in expression of endogenous IGF-IR correlated to the inhibition in the cell proliferation rate with cells from the C9 clone displaying the greatest decrease in cell proliferation rate while cells from the C8 clone had a slightly higher proliferation rate than cells from the C9 clone. Similar results have been demonstrated for other cancer cell types including human ovarian carcinoma cells and human glioblastoma cells, T98G .[37,38]

The abolishment of anchorage-independent growth in soft agar by cells from the C8 and C9 clones indicates that the transfromed phenotype has been reversed following treatement with antisense IGF-IR. We found that the antisense strategy was more effective in inhibiting growth of MDA-MB-435s cells in soft agar than in the cell proliferation assay. This is in agreement with a report that showed that the IGF-I receptor

has a more profound influence on the transformed phenotype than on cellular proliferation for several tumor cell lines including MCF-7 human breast cancer cells.[38] It is interesting to note that the abrogation of colony formation in soft agar does not always translate to complete inhibition of tumorigenesis *in vivo*. This could be due to the length of time the cells are studied since *in vivo* experiments are carried out over a period of several weeks or months versus *in vitro* experiments that are usually assessed after several days or a few weeks.

The IGF-IR has been reported to be an important factor in the regulation of apoptosis in mammalian cells. A decrease in the number of receptors causes massive apoptosis whereas overexpressed IGF-IRs protect cells from apoptosis.[28] Rat glioma cells incubated with antisense IGF-IR oligodeoxynucleotide displayed massive apoptosis *in vivo* within 24 hours after being placed in a biodiffusion chamber that was inserted in the subcutaneous tissue of syngeneic rats.[40] We have also demonstrated that the breast cancer cells treated with the antisense IGF-IR construct displayed avid apoptosis. Cells from both the C8 and C9 clones carrying antisense IGF-IR were shown to be apoptotic *in vitro* by DNA laddering. In addition cells from the C9 clone showed extensive apoptosis by flow cytometric analysis that correlated with the decreased cell proliferation rate and reduced expression of endogenous IGF-IR that was seen in the cells from the C9 clone.

We have also demonstrated that there was a significant delay in tumor formation and a dramatic reduction in tumor size when cells from the C8 and C9 clones carrying antisense IGF-IR were injected in the region of the mammary fat pad in nude mice. Indeed there were several animals injected with cells from the C8 clone that were tumor free at the time of sacrifice. We have previously reported that rat prostate cancer cells

treated with a construct carrying antisense IGF-IR have a delay in formation of tumors and significant inhibition of tumor growth in nude mice.[20] Furthermore, in a recent study we demonstrated that treatment of rat C6 glioblastoma cells with a transfection vector that was constructed to drive transcription of the homopurine (AG) sequence 3' to the termination codon of the IGF-IR that can form a potential triple helix resulted in dramatic inhibition of tumor growth *in vivo*.[21] In addition the down regulation of IGF-IR by either antisense or a potential triple helix approach resulted in up-regulation of the cell surface expression of major histocompatability complex I (MHC I) suggesting that their may be an immune system component involved in the suppression of tumorigenesis.[21] Indeed a study by Resnicoff et al.[14] demonstrated that the injection of C6 cells expressing antisense IGF-IR RNA into syngeneic animals provided protection against tumor induction by C6 wild type cells and in fact caused complete regression of established C6 wild-type tumors. Therefore, the mechanisms by which gene therapy strategies targeting IGF-IR inhibits growth of some tumor cells may be through a decrease in receptor number as well as by yet undefined immune components

We observed that although tumors formed in the mice injected with cells carrying antisense IGF-IR from the C8 and C9 clones there were no apparent metastases to the lungs after 11 weeks. Our results are in agreement with a report that demonstrated that cells from a highly metastatic murine hepatic carcinoma lost their metastatic phenotype when transfected with a plasmid vector expressing IGF-IR cDNA in the antisense orientation.[41] These investigators suggested that in cells expressing IGF-IR antisense RNA, the growth advantage in liver and lung is apparently lost. However, the results from our bioassay comparing tumor formation and metastases in nude mice to that in scid

beige mice raises some interesting questions about the role of the rudimentary immune system present in nude mice on the metastatic progression of cancer cells. We observed that all of the scid beige mice injected with MDA-MB-435s cells carrying the construct minus the antisense IGF-IR insert had metastases to the lungs after 11 weeks whereas there were no detectable lung metastases in the nude mice. This suggests that the rudimentary immune system present in nude mice played an important role in inhibiting the development of metastasis from the MDA-MB-435s breast cancer cells. Previous studies have reported that severe combined immunodeficient (scid) mice which lack functional T- and B-cells are better models for human tumor transplantation than nude mice.[42,43] These investigators demonstrated that the nude mouse displays a residual systemic immunoreactivity that is significantly higher than that of the scid mouse. Based on their findings we chose the scid beige mouse model for our study since these animals lack not only functional T- and B- cells but also are deficient in NK cells. Our data indicates that the scid beige mouse may be a more suitable animal model for studying metastatic progression in the MDA-MB-435s human breast cancer cells. We also showed that scid beige mice injected with cells from the C8 and C9 clones carrying antisense IGF-IR had a dramatically prolonged survival compared to animals injected with cells carrying the control construct. This finding indicates that IGF-IR plays an important role in the growth and progression of this human breast cancer cell line. Whether this will be the case for other types of breast cancer cells remains to be determined.

In conclusion our data has demonstrated that the IGF-IR plays a pivotal role in proliferation, transformation, tumorigenesis and growth progression in a metastatic

estrogen receptor negative human breast cancer cell line. Moreover, we have provided data that showed the importance of establishing and using the optimal animal model for studying the effects of down regulation of IGF-IR by antisense strategies under *in vivo* conditions. New approaches targeting the IGF-IR may provide an effective antimetastatic treatment strategy for patients that have progressive estrogen receptor negative breast cancer.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Department of Defense, DMAD17-95-1-5020 to Judith Ilan.

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FIGURE LEGENDS

Figure 1. Schematic representation of antisense IGF-IR vector (IGF-IRAS) and the sense IGF-IR vector. (A) The insert for the antisense IGF-IR construct was assembled using the 697 bp human IGF-IR fragment in the antisense orientation. The final insert following recloning was 748 bp. (B) The insert for the sense construct was assembled using the 697 bp human IGF-IR fragment in the sense orientation. The final insert following recloning was 787 bp. Both constructs also contain sense neomycin. Transcription of IGF-IR antisense, IGF-IR sense and neomycin sense are under the control of the constitutive cytomegalovirus (CMV) promoter. The construct used for control transfections lacked the antisense IGF-IR and sense IGF-IR inserts. Amp R, ampicillin resistance gene; Neo R, Neomycin resistance gene; SV40pA, Simian virus 40 polyadenylation signal; ColE1, ColE1 origin of replication; H, *Hin*dIII; E, *Eco*RI; P,*Pst*I; N, *Not*I; X, *Xho*I.

Figure 2. Expression of antisense IGF-IR transcripts and endogenous IGF-IR transcripts in cultured MDA-MB-435s human breast cancer cells carrying the control vector (lane 1), in cells from the C8 (lane 2) and C9 (lane 3) clones carrying the antisense IGF-IR vector. (A) Poly A^+ RNA (5μg) was used per lane, hybridized with the ^{32}P-labeled 0.7 kb IGF-IR cDNA fragment and the membrane was exposed to an X-ray film for 12 hr at -80° C. The molecular sizes of IGF-IR antisense (2 kb) and the endogenous IGF-IR transcripts (11 kb and 7 kb) are shown on the right. (B) Rehybridization of the same filter was performed with a cDNA probe for chicken β-actin. The difference in the expression of mRNA transcripts was calculated relative to the β-actin standards. (C) Results of analysis for endogenous IGF-IR in cells carrying the control vector (*black bar*) and in cells from the C8 (*stippled bar*) and C9 (*white bar*) clones carrying IGF-IR antisense are shown in the bar graph. The numbers under the bar graph correspond to the numbers used in A and B.

Figure 3. Effect of treatment with an IGF-IR antisense construct on the proliferation rate of MDA-MB-435s human breast cancer cells. Cell proliferation was measured using a MTT based assay. Data points, mean of 6 wells ± SE. (A) Treatment of MDA-MB-435s cells with an antisense IGF-IR construct inhibits cell proliferation in two clonal populations, C8 (pRcII/IGF-IRASC8, *triangles*) and C9 (pRcII/IGF-IRASC9, *circles*) compared to cells transfected with the construct minus the IGF-IR inserts (pRcII/CMV, *diamonds*). (B) Treatment of MDA-MB-435s cells with a sense IGF-IR construct (pRcII/IGF-IRS, *squares*) as a positive control does not alter the cell proliferation rate when compared to cells transfected with the vector minus the IGF-IR inserts (pRcII/CMV, *diamonds*). However, cells from the C9 clone carrying the antisense IGF-IR construct (pRcII/IGF-IRASC9, *circles*) display significant inhibition in the proliferation rate when cultured under the same conditions.

Figure 4. Soft agar colonization of MDA-MB-435s human breast cancer cells. Assays were performed in parallel on all cells under identical culture conditions. (A and B) Photographs from a soft agar assay carried out on cells from the C8 and C9 clones, respectively, carrying the antisense IGF-IR construct. (C) Photograph of cells carrying the sense IGF-IR construct. (D) Photograph of cells carrying the construct minus the IGF-IR inserts.

Figure 5. Treatment of MDA-MB-435s human breast cancer cells with antisense IGF-IR induces internucleosomal DNA cleavage under serum withdrawal conditions. Cells from the C8 and C9 clones carrying antisense IGF-IR exhibited DNA laddering on day 3 (Lanes 3 and 5, respectively) with more prominent laddering occurring on day 5 (Lanes 4 and 6, respectively). There was no apparent laddering in the cells carrying the vector minus the IGF-IR inserts on either day 3 or day 5 (Lanes 1 and 2, respectively). The cells were harvested and the DNA extracted, labeled and electrophoresed as described in "Methods". This result was reproduced in four experiments. The molecular marker λ-DNA, cleaved with *Eco*RI and *Hind*III ladder on the left was used to determine size in bp.

Figure 6. Transfection of MDA-MB-435s human breast cancer cells with an antisense IGF-IR construct results in 3'-OH DNA strand breaks. Following harvest, fixation, and permeabilization the TUNEL technique, apoptosis detection system, fluorescein was used to label the cells for analysis by flow cytometry. 1 x 10^6 cells were prepared for analysis per determination. Histograms show DNA strand breaks (TUNEL labeling) for cells from the C9 clone (A) and for cells carrying the construct minus the IGF-IR inserts (B).

Figure 7. Tumor growth in nude mice. Mice injected with cells carrying antisense IGF-IR from the (A) C8 cell clone or the (B) C9 cell clone display undetectable tumor growth at 11 weeks post injection. (C) Large tumors were apparent in mice injected with cells carrying the construct minus the IGF-IR inserts.

Figure 8. Delayed onset and suppression of tumorigenesis by MDA-MB-435s human breast cancer cells treated with an antisense IGF-IR construct. Tumor volumes were measured weekly for 11 weeks with calipers and tumor volume was calculated. Tumor growth of cells from the C8 clone (*triangles*) and the C9 clone (*circles*) carrying antisense IGF-IR was dramatically suppressed compared to cells carrying the construct minus the IGF-IR inserts (*diamonds*). Each data point represents the mean ± SE for each group of mice.

Figure 9. Suppression of tumorigenesis in nude and scid beige mice injected with MDA-MB-435s human breast cancer cells carrying antisense IGF-IR. Female mice were injected s.c. in the region of the thoracic mammary fat pad with cells from the C8 (*stippled bars*) and C9 (*white bars*) clones as well as with cells carrying the construct minus the IGF-IR inserts (*black bars*). The mice were killed 11 weeks postinjection, the tumors were excised and the tumor weights were determined. Data represents the mean ± SE for each group of mice.

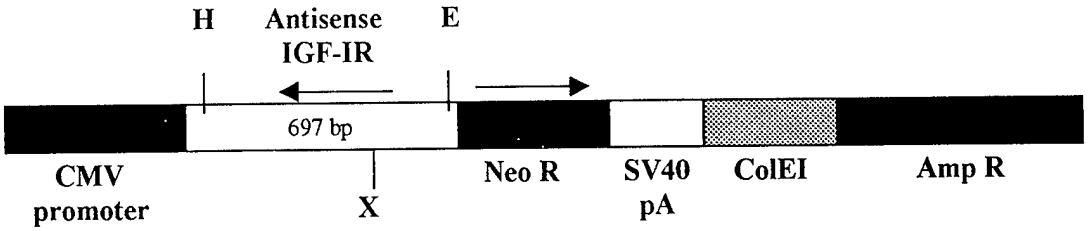
Figure 10. Survival curve for MDA-MB-435s human breast cancer cells from the C8 and C9 clones carrying antisense IGF-IR injected into scid beige mice. The animals injected with cells from the C8 clone (*long dashes*) and the C9 clone (*short dashes*) survived significantly longer that mice injected with cell carrying the construct minus the IFG-IR inserts (*solid lines*).

Table I. Number of animals with tumors and metastases to lung (parentheses) following treatment of MDA-MB-435s cells with an antisense IGF-IR construct.

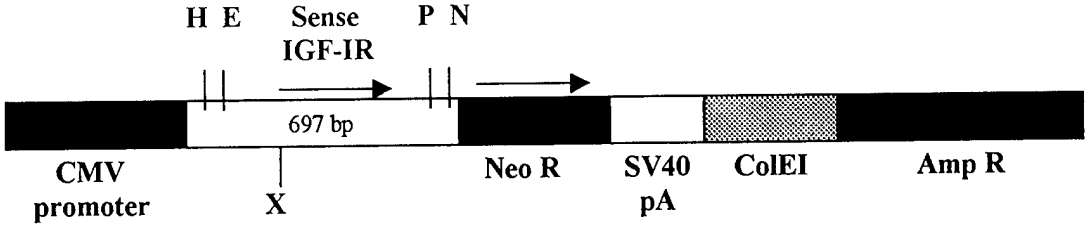
Treatment	Nude mice	Scid beige mice
PRcII/CMV	0/5 (0)	4/4 (10)*
PRcII/IGFIRASC8	0/5 (0)	0/3 (0)
PRcII/IGFIRASC9	0/5 (0)	0/5 (0)

*Denotes scid beige mice injected with cells carrying the construct minus the IGF-IR inserts.

A


H
Antisense
IGF-IR
E
697 bp
CMV
promoter
X
Neo R
SV40
pA
ColEI
Amp R

B


H E
Sense
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P N
697 bp
CMV
promoter
X
Neo R
SV40
pA
ColEI
Amp R

Fig. 1

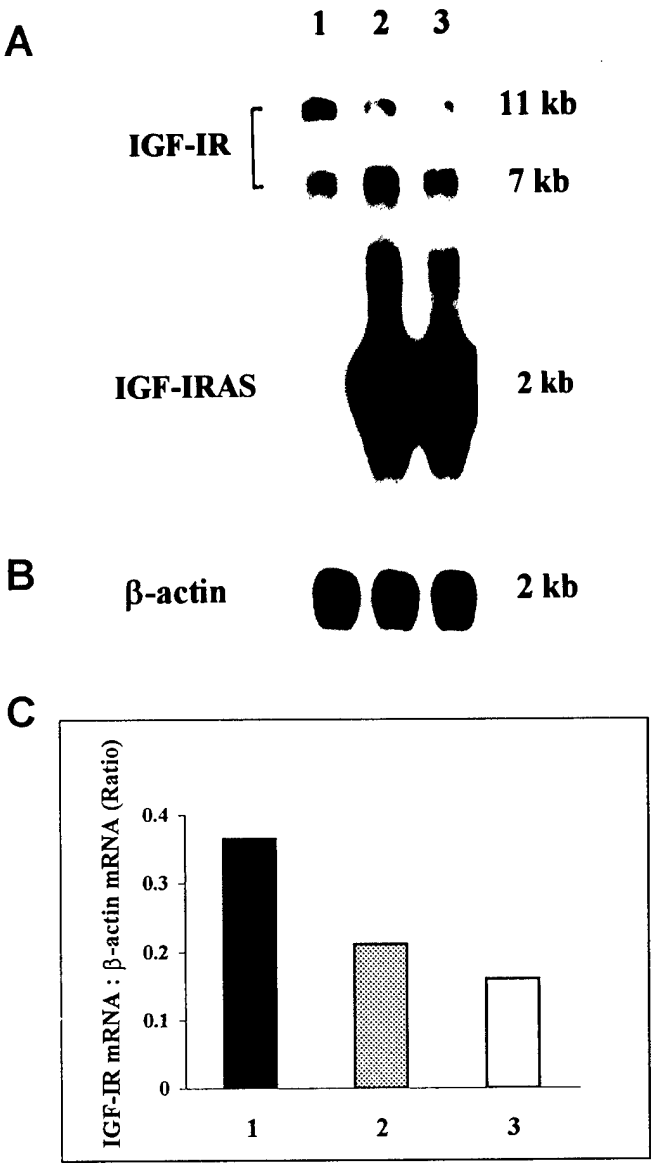
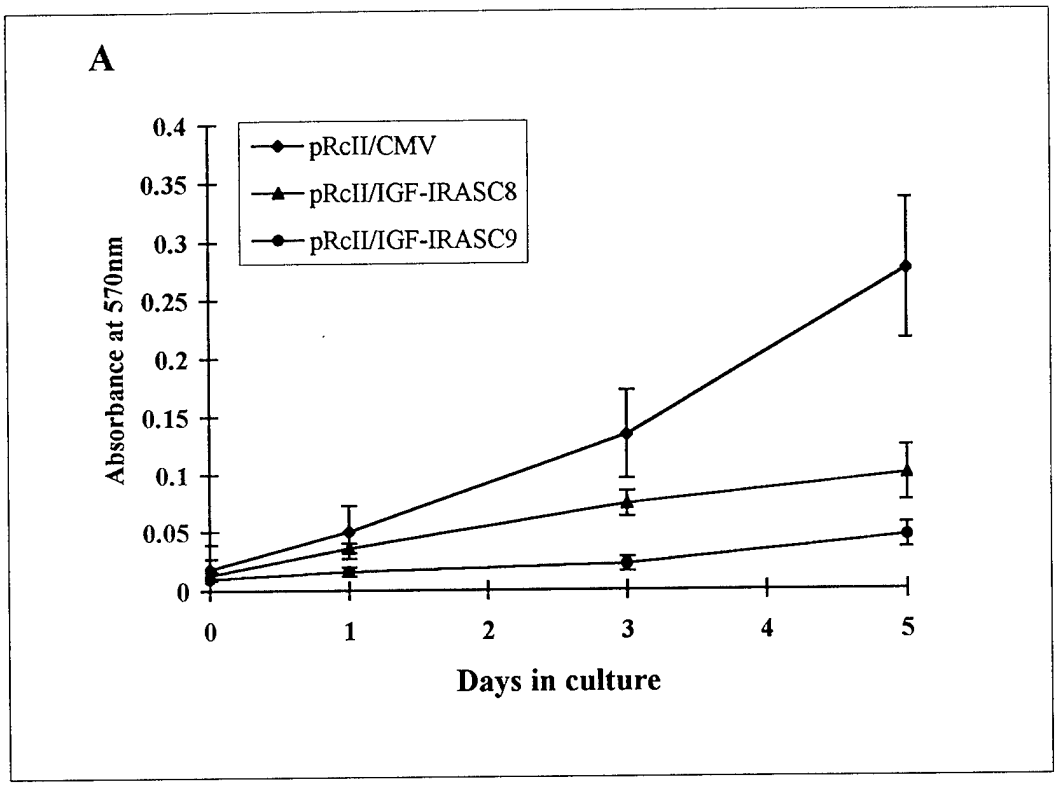

A
1 2 3
IGF-IR
11 kb
7 kb
IGF-IRAS
2 kb
B
β-actin
2 kb
C
IGF-IR mRNA : β-actin mRNA (Ratio)
0.4
0.3
0.2
0.1
0
1
2
3

Fig.2


A
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Absorbance at 570nm
pRcII/CMV
pRcII/IGF-IRASC8
pRcII/IGF-IRASC9
0
1
2
3
4
5
Days in culture

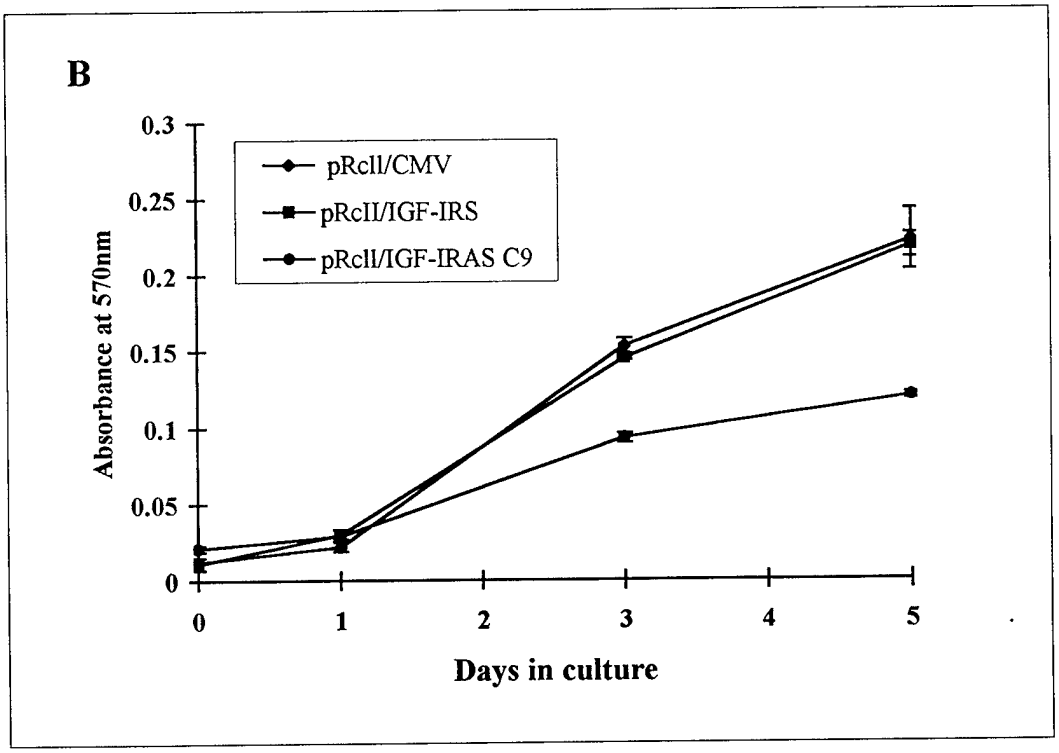

B
0.3
0.25
0.2
0.15
0.1
0.05
0
Absorbance at 570nm
pRcll/CMV
pRcII/IGF-IRS
pRcll/IGF-IRAS C9
0
1
2
3
4
5
Days in culture

Fig.3

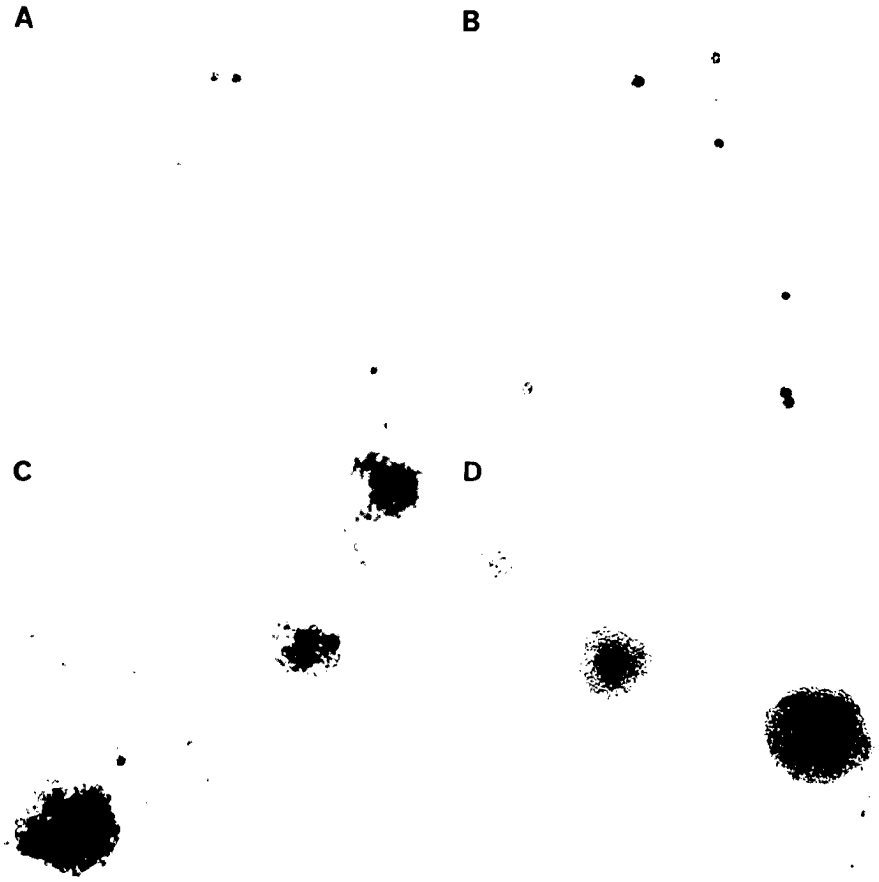


Fig.4

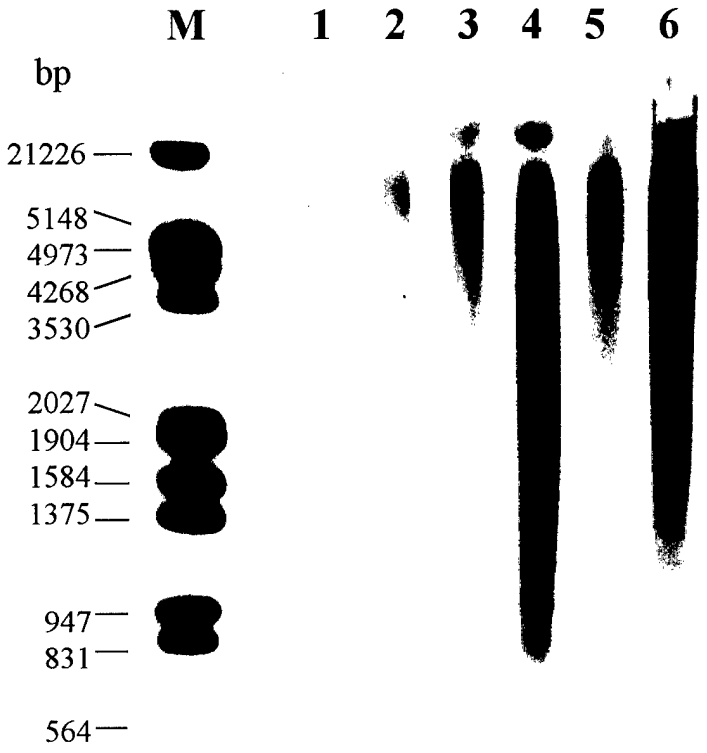

M
1
2
3
4
5
6
bp
21226
5148
4973
4268
3530
2027
1904
1584
1375
947
831
564

Fig.5

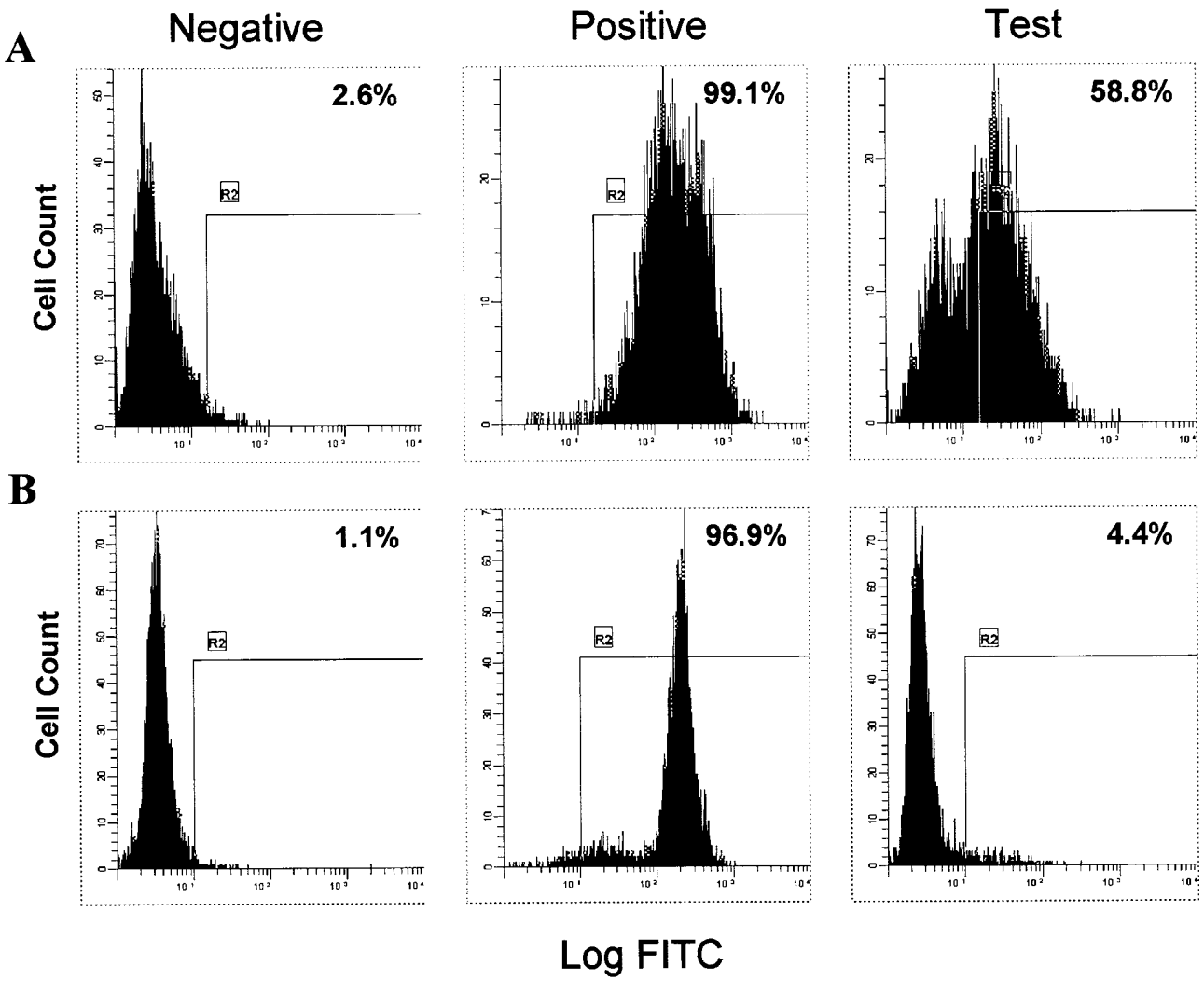

A
Negative
Positive
Test
2.6%
99.1%
58.8%
R2
Cell Count
B
1.1%
96.9%
4.4%
R2
Cell Count
Log FITC

Fig.6

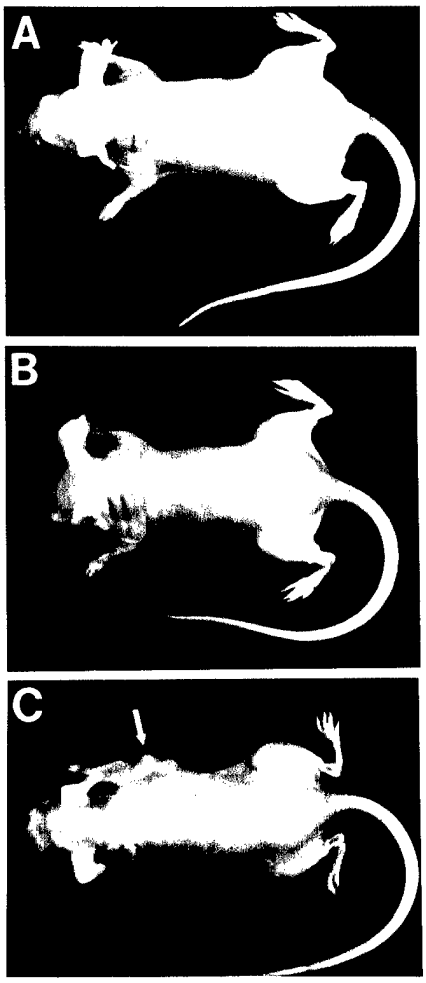


Fig.7

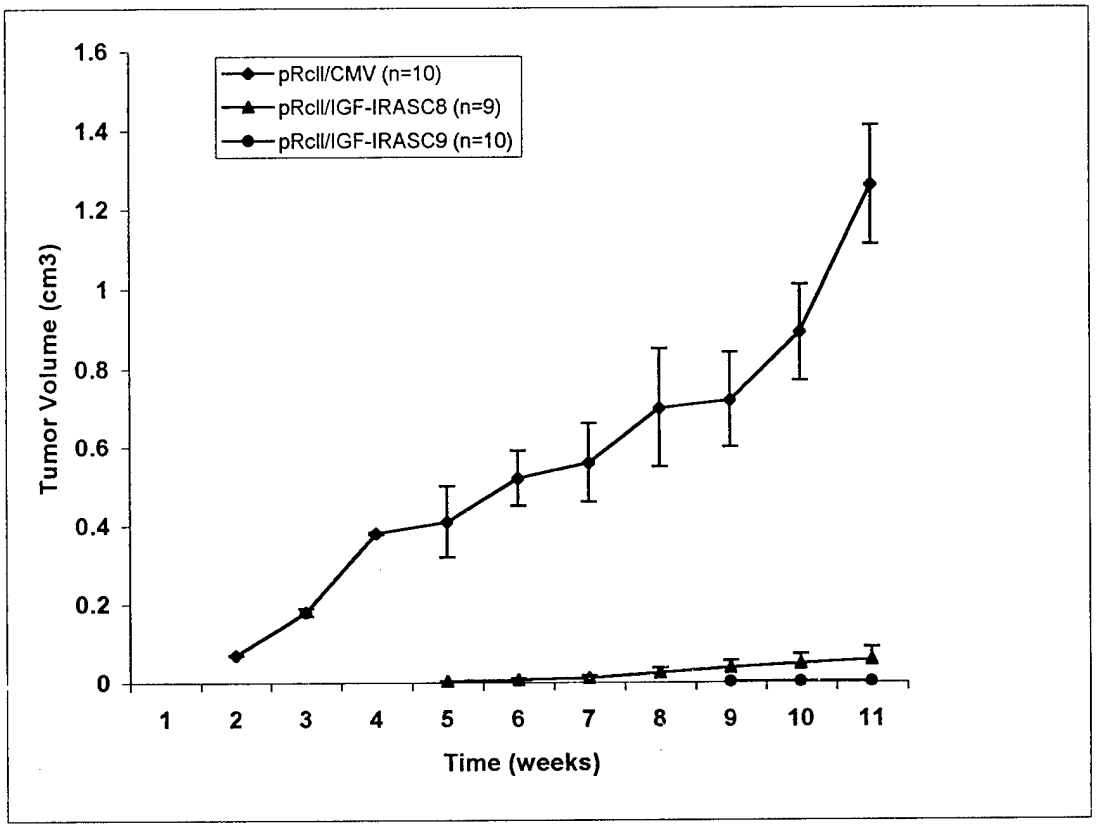

pRcll/CMV (n=10)
pRcll/IGF-IRASC8 (n=9)
pRcll/IGF-IRASC9 (n=10)
Tumor Volume (cm3)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1
2
3
4
5
6
7
8
9
10
11
Time (weeks)

Fig.8

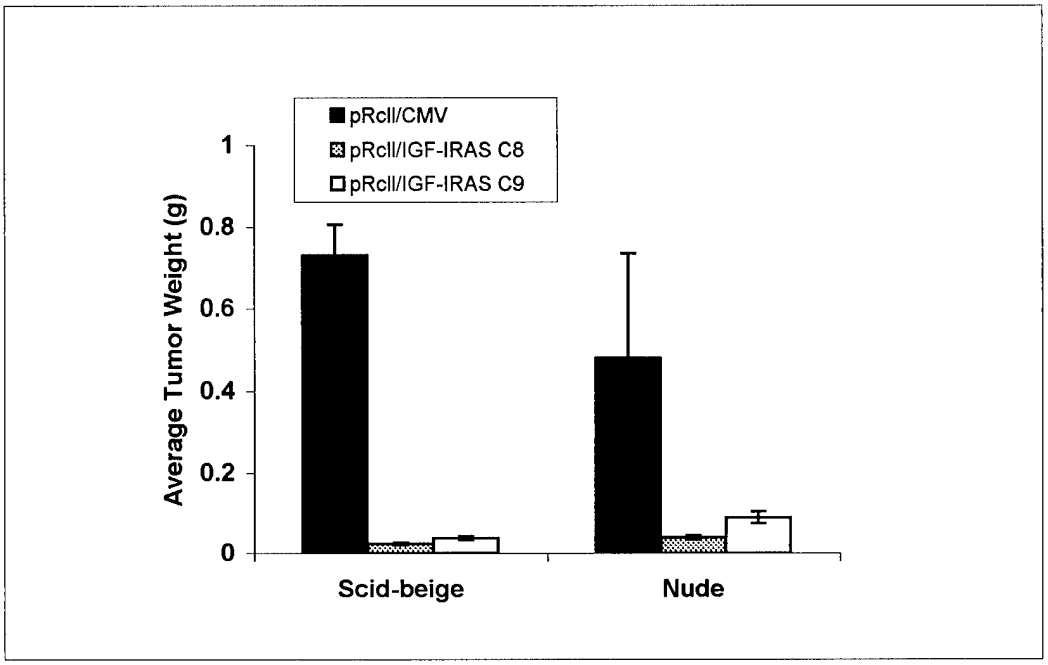

pRcII/CMV
pRcII/IGF-IRAS C8
pRcII/IGF-IRAS C9
Average Tumor Weight (g)
1
0.8
0.6
0.4
0.2
0
Scid-beige
Nude

Fig.9

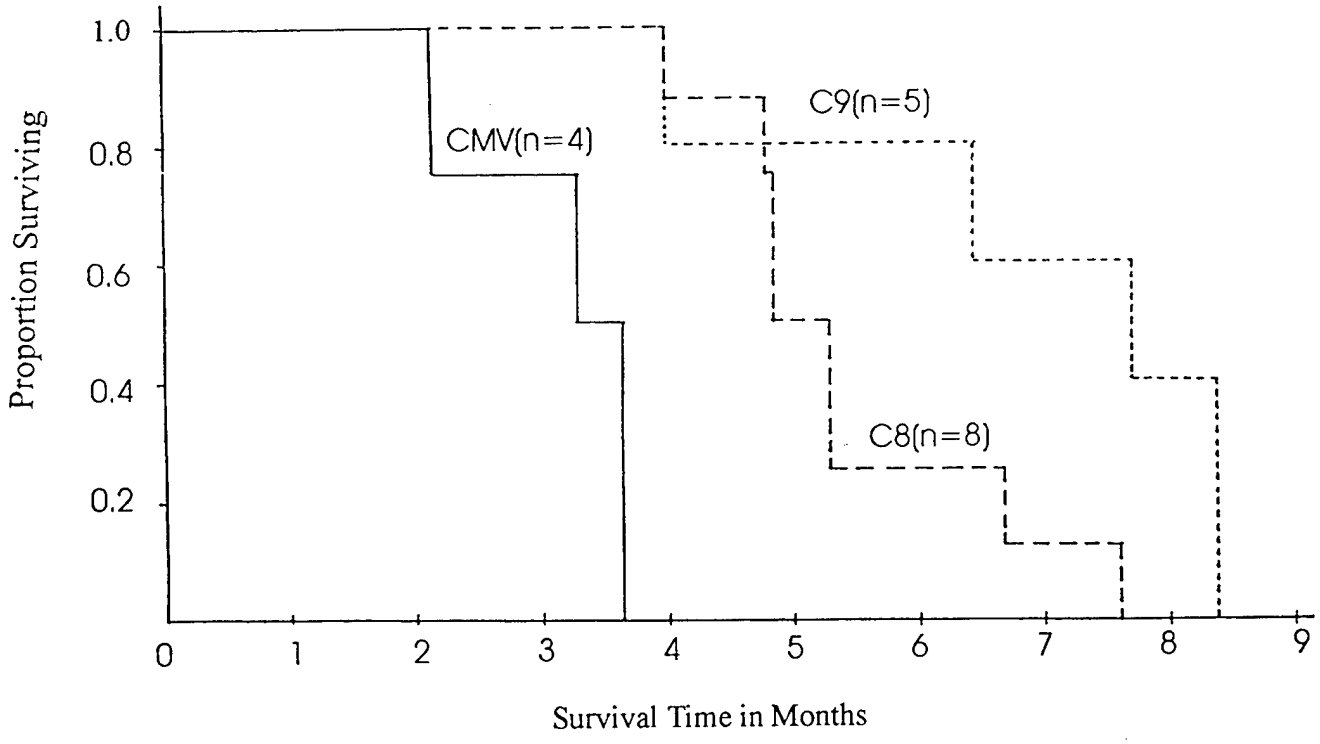

1.0
0.8
0.6
0.4
0.2
Proportion Surviving
CMV(n=4)
C9(n=5)
C8(n=8)
0
1
2
3
4
5
6
7
8
9
Survival Time in Months

Fig.10

FINAL REPORT BIBLIOGRAPHY OF PUBLICATIONS AND PERSONNEL:

Publications:

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